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The wide-ranging phenotypes of ergosterol biosynthesis mutants, and implications for microbial cell factories

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Abstract

Yeast strains have been used extensively as robust microbial cell factories for the production of bulk and fine chemicals, including biofuels (bioethanol), complex pharmaceuticals (antimalarial drug artemisinin and opioid pain killers), flavours, and fragrances (vanillin, nootkatone, and resveratrol). In many cases, it is of benefit to suppress or modify ergosterol biosynthesis during strain engineering, for example, to increase thermotolerance or to increase metabolic flux through an alternate pathway. However, the impact of modifying ergosterol biosynthesis on engineered strains is discussed sparsely in literature, and little attention has been paid to the implications of these modifications on the general health and well-being of yeast. Importantly, yeast with modified sterol content exhibit a wide range of phenotypes, including altered organization and dynamics of plasma membrane, altered susceptibility to chemical treatment, increased tolerance to high temperatures, and reduced tolerance to other stresses such as high ethanol, salt, and solute concentrations. Here, we review the wide-ranging phenotypes of viable *Saccharomyces cerevisiae* strains with altered sterol content and discuss the implications of these for yeast as microbial cell factories.

KEYWORDS

fecosterol, endocytosis, episterol, plasma membrane, sterol, stress, thermotolerance

1 | INTRODUCTION

Fungal sterols are steroidal structures composed of four rigid rings, with a hydroxyl group at carbon 3 and an aliphatic tail at carbon 17. Ergosterol is the predominant sterol in yeast and has been reported to comprise 12 mol% of the *Saccharomyces cerevisiae* lipidome, which encompasses glycerophospholipid, sphingolipid, and sterol species (Ejsing et al., 2009). Within yeast cells, ergosterol is mainly located at the plasma membrane (PM), secretory vesicles, and lipid particles (Sokolov, Trushina, Severin, & Knorre, 2019; Zinser et al., 1991; Zinser, Paltauf, & Daum, 1993).

Strains lacking or producing alternative sterols exhibit a range of phenotypes. In recent years, yeast strains have been engineered as microbial cell factories for a wide variety of heterologous products, and a common approach to increase strain productivity is to suppress native pathways that compete for substrate. In many cases, particularly for terpene engineering, ergosterol biosynthesis has been suppressed in production strains (Moses et al., 2014; Paddon et al., 2013). It is therefore industrially relevant to consider the impact of altered sterol compositions on yeast health, in order to optimize production strains and processes.

This article provides an overview of ergosterol biosynthesis in the budding yeast *S. cerevisiae*, reviews the phenotypes of viable strains

with altered sterol compositions, and discusses the implications of these for microbial cell factories.

2 | ERGOSTEROL BIOSYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

The biosynthesis of ergosterol from acetyl-CoA is often grouped into three modules: (a) the mevalonate biosynthesis module, (b) the farnesyl pyrophosphate (FPP) biosynthesis module, and (c) the ergosterol biosynthesis module (Figure 1; reviewed in Hu et al., 2017). The ergosterol biosynthetic enzymes are localized in different subcellular compartments of the yeast cell, with Erg proteins of the first, second, and third modules predominantly located in the mitochondria (Isamu, Jun, Hiroshi, & Hirohiko, 1973), cytoplasm and endoplasmic reticulum (ER; Nishino, Hata, Taketani, Yabusaki, & Katsuki, 1981), respectively.

The mevalonate biosynthesis module consists of three steps and begins with the condensation of two acetyl-CoA molecules to acetoacetyl-CoA, catalysed by acetyl-CoA acetyltransferase (Erg10). The hydroxymethylglutaryl-CoA synthase enzyme (Erg13) then

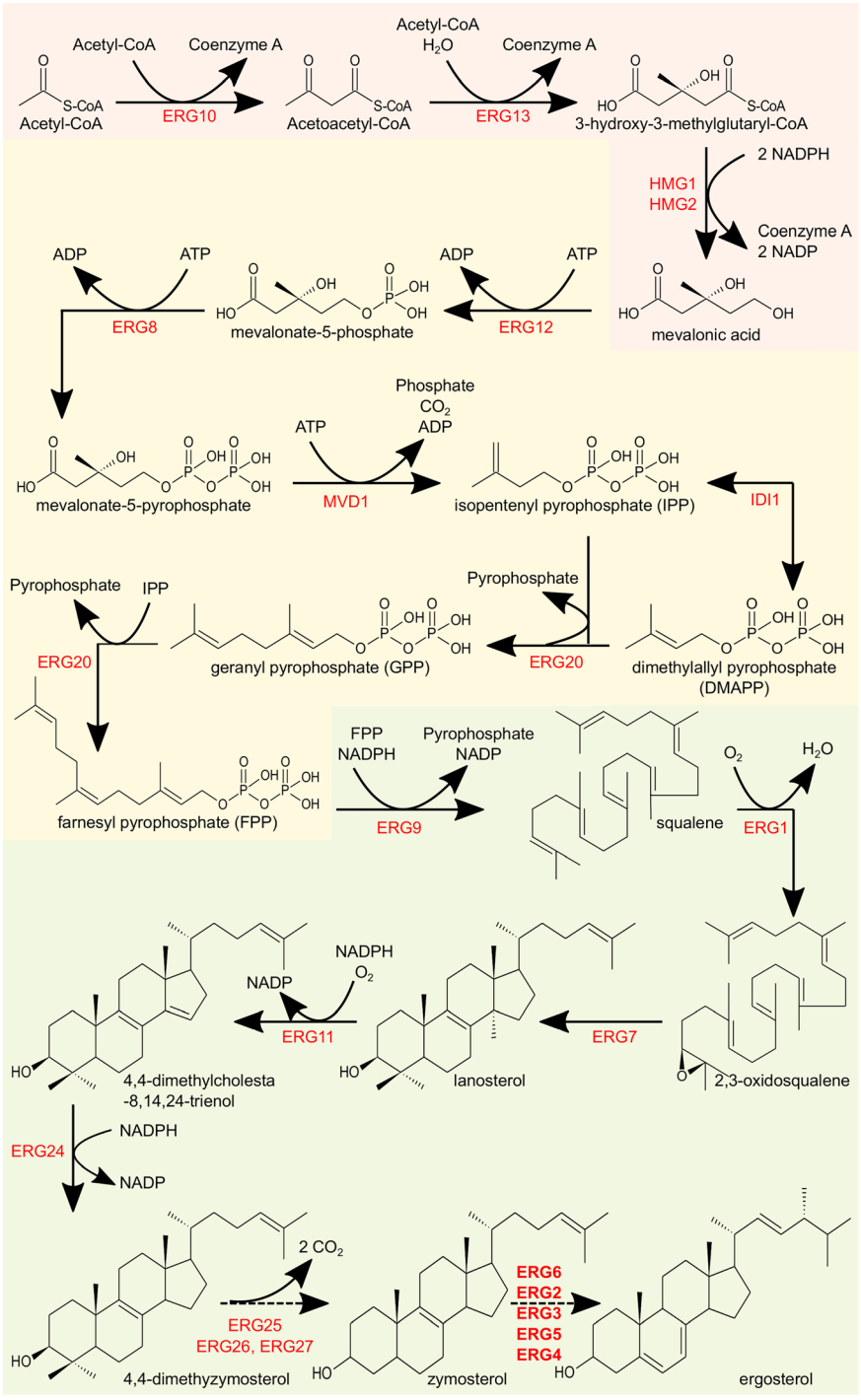

FIGURE 1 Ergosterol biosynthesis in *Saccharomyces cerevisiae* Reactions of the mevalonate biosynthesis module (red), farnesyl pyrophosphate (FPP) biosynthesis module (yellow) and ergosterol biosynthesis module (green). The enzyme names are given in red. Enzymes which are non-essential for growth in aerobic conditions are emboldened

catalyses the condensation of a third molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is reduced by HMG-CoA reductase (Hmg1 and Hmg2) to mevalonic acid.

The second module involves a series of phosphorylation reactions and begins with the phosphorylation of mevalonate by mevalonate kinase (Erg12) to phosphomevalonate, which is further phosphorylated to mevalonate-5-pyrophosphate by phosphomevalonate kinase (Erg8). Mevalonate pyrophosphate decarboxylase (Mvd1/Erg19) subsequently catalyses the decarboxylation of mevalonate-5-pyrophosphate to isopentenyl pyrophosphate (IPP). The 5-carbon prenyl phosphate, IPP is then isomerised to dimethylallyl pyrophosphate by IPP isomerase (Idi1). The condensation of the isomers IPP and dimethylallyl pyrophosphate result in the formation of geranyl pyrophosphate, which condenses with another molecule of IPP to form FPP. The above two condensation reactions between the pyrophosphates are catalysed by FPP synthase (Erg20).

The ergosterol biosynthesis module begins with the condensation of two FPP molecules to the linear 30-carbon squalene by squalene synthase (Erg9). Squalene is then epoxidized by squalene epoxidase (Erg1) to 2,3-oxidosqualene, which is cyclised to the four-ring triterpene lanosterol by lanosterol synthase (Erg7). Lanosterol, the dedicated precursor for fungal sterol biosynthesis, is then demethylated by lanosterol C-14 demethylase (Erg11) to 4,4-dimethylcholesta-8,14,24-trienol, which is reduced by sterol C-14 reductase (Erg24) to 4,4-dimethylzymosterol. The oxidoreductases sterol C-4 methyl oxidase (Erg25), sterol C-4 decarboxylase (Erg26), and sterol 3-keto reductase (Erg27) then sequentially catalyse the conversion of 4,4-dimethylzymosterol to zymosterol, the first intermediate of the ergosterol biosynthesis pathway that can be incorporated into cell membranes. The final reactions in this module require an oxygen-rich environment and generate sterol intermediates that can be built into the yeast cell membrane (Figure 2). Zymosterol is methylated by sterol C-24 methyltransferase (Erg6) to fecosterol, which is isomerised by sterol C-8 isomerase (Erg2) to episterol. In two subsequent desaturation reactions episterol is converted to ergosta-5,7,22,24(28)-tetraenol via ergosta-5,7,24(28)-trienol by the action of sterol C-5 desaturase (Erg3) and C-22 desaturase (Erg5). The final reaction of this pathway is the reduction of ergosta-5,7,22,24(28)-tetraenol to ergosterol by sterol C-24 reductase (Erg4).

The ergosterol biosynthesis pathway is energy intensive, with at least 24 molecules of ATP and 16 molecules of NADPH estimated to be required for the *de novo* synthesis of one molecule of ergosterol (Hu et al., 2017). In addition, oxygen is an essential cofactor for several enzymes, which makes the ergosterol biosynthesis pathway dependent on the availability of environmental oxygen (Galea & Brown, 2009). However, in aerobic conditions, yeast cells can overproduce ergosterol, an excess of which is cytotoxic to yeast cells in the free form (Liu, Xia, Nie, Wang, & Deng, 2019). Therefore, stringent regulation and maintenance of cellular sterol homeostasis are critical. To attenuate the excessive sterol pool, yeast cells either secrete sterol acetates into the extracellular matrix (Hu et al., 2017) or store steryl esters in lipid particles (Taylor & Parks, 1978). Under anaerobic conditions, sterol uptake becomes essential for growth and is obtained either from esterified sterol reserves that can be readily interconverted to free sterols, or from the environment using an import process mediated by ATP-binding sterol transporters Aus1 and Prd11 that are repressed in the presence of oxygen (reviewed in Hu et al., 2017; Liu et al., 2019). The regulation of ergosterol biosynthesis is tightly controlled at several check points and accomplished by feedback mechanisms at the level of transcription, translation, and post-translation, including the inhibition of key enzymes by ergosterol pathway intermediates and gene activation by transcription factors Upc2, Ecm22, Rox1, and Mot3 (reviewed in Liu et al., 2019).

3 | STEROL PROFILES OF VIABLE ERGOSTEROL BIOSYNTHESIS MUTANTS

Most of the studies in this review report the phenotypes of viable ergosterol biosynthesis mutants, which vary significantly in sterol composition. Yeast strains deficient in Erg6, Erg2, Erg3, Erg5, or Erg4 are able to grow under routine laboratory conditions (Palermo, Leak, Tove, & Parks, 1997). The remaining *ERG* genes are classified as essential, although *erg24Δ* strains are able to grow under specific conditions, such as in defined synthetic media but not yeast extract peptone-based media under aerobic conditions (Crowley, Smith, Leak, & Parks, 1996; Lorenz & Parks, 1992; Palermo et al., 1997).

At the PM of wild type yeast, ergosterol is the predominant sterol, with minor amounts of zymosterol (Zinser et al., 1993). Yeast strains with deletions of genes in the late steps of ergosterol biosynthesis accumulate sterols that differ from ergosterol in the number and position of double bonds in the B-ring and the side chain of the sterol molecule. Sterol profiling suggests that Erg enzymes catalysing the last five steps of ergosterol biosynthesis have reduced substrate specificity and can accept a broad range of similar sterol structures. As a result, mutants of late *ERG* genes accumulate mixtures of sterols including the precursors of the enzymes they lack. The most abundant sterols in these yeast mutants are depicted in Figure 2, and the potential catalytic activities that lead to their synthesis are discussed here.

In strains with nonfunctional Erg6, the sterol side chain is not methylated and unsaturation at Carbon 24 is retained, resulting in the accumulation of zymosterol (substrate of Erg6), cholesta-7,24-dienol (catalytic activity of Erg2 on zymosterol), cholesta-5,7,24-trienol (catalytic activity of Erg2 and Erg3 on zymosterol), and cholesta-5,-7,22,24-tetraenol (catalytic activity of Erg2, Erg3, and Erg5 on zymosterol; Heese-Peck et al., 2002). Lack of Erg2 isomerase activity results in the accumulation of fecosterol (substrate of Erg2), egosta-8-enol (catalytic activity of Erg4 on fecosterol), and ergosta-5,-8,22-trienol (catalytic activity of Erg3, Erg5, and Erg4 on fecosterol) (Abe & Hiraki, 2009). In *erg3Δ* yeast, accumulation of episterol (substrate of Erg3), ergosta-7,22-dienol (catalytic activity of Erg5 and Erg4 on episterol), and ergosta-7-enol (catalytic activity of Erg4 on episterol) has been reported (Heese-Peck et al., 2002). The *erg5Δ* strain, on the other hand, accumulates ergosta-5,7,24(28)-trienol (substrate of Erg5) and ergosta-5,7-dienol (catalytic activity of Erg4 on ergosta-5,7,24(28)-trienol) (Barton, Corrie, Bard, & Woods, 1974; Sun

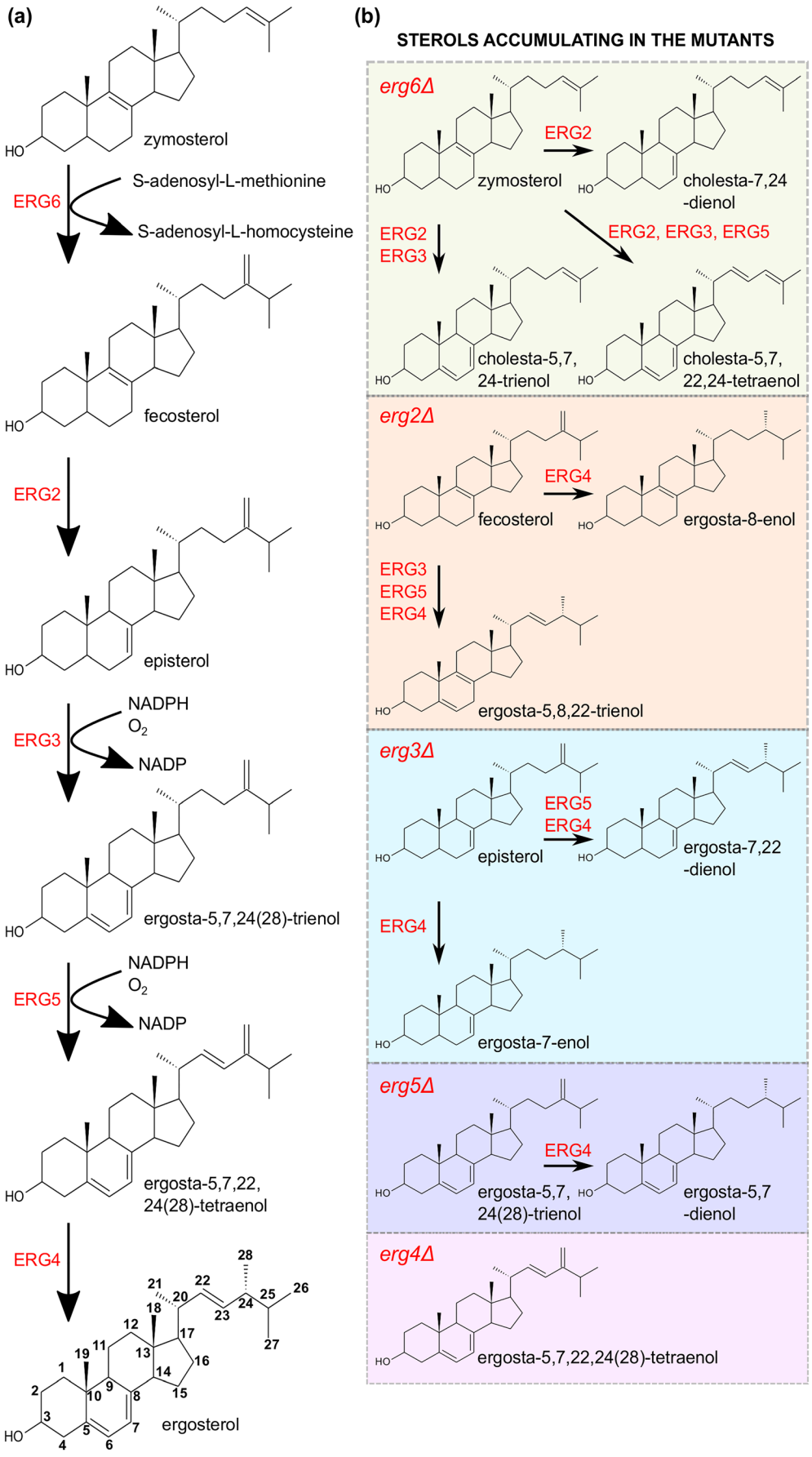

FIGURE 2 **Sterols accumulating in *ergΔ* strains (a)** Reactions catalysed by enzymes in the late steps of the ergosterol biosynthesis pathway, in the order which they are typically depicted. **(b)** Sterols reported to accumulate in yeast cells lacking Erg6, Erg2, Erg3, Erg5 or Erg4

et al., 2013), whereas the *erg4Δ* strain accumulates ergosta-5,7,22,24 (28)-tetraen-3-ol, the direct precursor of ergosterol (Aguilar et al., 2010).

Small changes in sterol methyl groups and positions of unsaturation in the ring skeleton or aliphatic tail, significantly impact the dynamics of lipid bilayers (Ranadive & Lala, 1987; Shahedi, Orädd, & Lindblom, 2006). Sterol structure impacts van der Waals interactions with other lipid bilayer components and influences the packing effects of sterols when included in model membranes. Notably, small variations in saturation within the stiff sterol ring structure can change the orientation of sterol rings, rendering a sterol flat (for example, ergosterol) or bended (for example, fecosterol and episterol) (Shahedi et al., 2006). These differences significantly alter the biophysical properties of the membrane (Ranadive & Lala, 1987). In the following section, we explore the impact of altered sterol composition on yeast membranes in further detail.

4 | PHENOTYPES OF ERGOSTEROL BIOSYNTHESIS MUTANTS

4.1 | Plasma membrane structure

PM is composed of glycerophospholipids, sphingolipids, sterols, and a plethora of proteins, with roles in transport, signalling and organisation of the cytoskeleton (van der Rest et al., 1995). Studies using model membranes have indicated that sterols have a key role in maintaining PM dynamics (Figure 3; Arora, Raghuraman, & Chattopadhyay, 2004; Dufourc, 2008; Hsueh et al., 2007; Hsueh, Gilbert, Trandum, Zuckermann, & Thewalt, 2005; Low, Rodriguez, & Parks, 1985; Shrivastava & Chattopadhyay, 2007; Soubias, Jolibois, Massou, Milon, & Réat, 2005). In particular, sterols are considered to interact strongly with the long and highly saturated acyl chains of sphingolipids (Ahmed, Brown, & London, 1997; Wattenberg & Silbert, 1983). In the "lipid raft" model for PM structure, microdomains rich in sterol and sphingolipid are considered to form detergent-insoluble rafts of liquid ordered phase, surrounded by bulk liquid disordered phase. The reliability of using model membranes and detergent solubility studies to define PM structure has been controversial (reviewed in Hancock, 2006; Lichtenberg, Goñi, & Heerklotz, 2005; Munro, 2003); however in yeast, studies using green fluorescent protein (GFP)-tagged PM proteins have provided growing evidence for lateral compartmentation of the PM, into domains of distinct protein and possibly lipid combinations (reviewed in Merzendorfer & Heinisch, 2013). The first type of domain to be described was termed MCC (for membrane compartment of Can1), to describe the patch-like codistribution of the proton-dependent permeases Can1, Fur4, Tat2, and other proteins. The second to be described was termed MCP (for membrane compartment of Pma1), which describes the network-like distribution of the H^+-ATPase Pma1, which is strictly distinct from the MCC patches. Further domains that have been described are MCT (for membrane compartment of TORC2), and CWI (for cell wall integrity; reviewed in Merzendorfer & Heinisch, 2013).

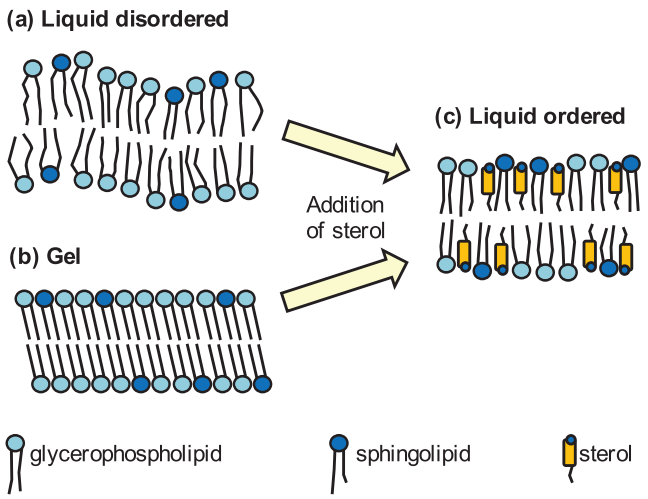

FIGURE 3 **Membrane phases (a)** Simplified schematic of a lipid bilayer in 'liquid disordered' phase. In this highly fluid state, lipids exhibit irregular packing with increased disorder and lateral mobility. **(b)** Below melting temperature, lipid bilayers pack tightly in a solid-like phase known as 'gel phase'. This impedes the lateral movement of lipids. **(c)** When sterols are included in model membranes, bilayers form a 'liquid ordered phase' in which acyl chains are packed more closely than in liquid disordered phase, but retain lateral mobility

Viable ergosterol biosynthesis mutants (henceforth *ergΔ*) have exhibited altered glycerophospholipid and sphingolipid profiles, changes in PM dynamics, and changes in protein compartmentation at the PM. For example, Sharma (2006) reports *erg2Δ* and *erg3Δ* strains to have decreased phosphatidylcholine content and *erg6Δ* to have increased phosphatidylcholine and phosphatidylserine content. Meanwhile, Guan et al. (2009) report substantial changes in sphingolipid profiles between *ergΔ* mutants.

The physical properties of PM in *ergΔ* strains have been studied by measuring the time-resolved fluorescence anisotropy of the fluorescent dye trimethylammonium-diphenylhexatriene (TMA-DPH), which anchors in the outer leaflet of the PM. In a study by Abe and Hiraki (2009), with the exception of *erg5Δ*, viable *ergΔ* strains exhibited accelerated rotation of TMA-DPH in the PM; the rotational diffusion coefficients were significantly higher than wild type in the order *erg2Δ* > *erg6Δ* > *erg3Δ* > *erg4Δ*. The *erg2Δ*, *erg6Δ*, and *erg4Δ* strains also exhibited a decrease in order compared to wild type, with *erg2Δ* exhibiting the strongest deviation from the wild type. Overall, this study indicated decreased rigidity of the PM in *ergΔ* strains, with an increase in the occurrence of voids, particularly for the *erg2Δ* strain. The *erg2Δ* and *erg6Δ* strains accumulate sterols with unsaturation in carbon position 8(9), which differs considerably from the carbon 7(8) unsaturation in ergosterol (Figure 2). These altered sterols could account for the striking differences in PM dynamics.

A number of studies have indicated that sterols have a role in maintaining the compartmentation of PM proteins. For example, in a genome-wide screen for genes affecting the colocalisation of MCC domain proteins, Grossmann et al. (2008) identified that loss of Erg2, Erg24, or Erg6 strongly disrupted MCC formation. Staining of

S. cerevisiae cells with filipin (a fluorescent polyene that interacts with 3′-β-sterols) has indicated that MCC domains are rich in ergosterol (Grossmann, Opekarová, Malinsky, Weig-Meckl, & Tanner, 2007), and the filipin staining of MCC domains was not as distinct as wild type in *erg2Δ*, *erg24Δ*, or *erg6Δ* strains, indicating reduced compartmentation of sterols (Grossmann et al., 2008). Notably, Jin, McCaffery, and Grote (2008) suggest that filipin might only bind free ergosterol, as a strain with approximately 50% reduction in sphingolipid and a modest reduction in ergosterol content, exhibits bright uniform staining around the PM. However, it is also possible that sterol distribution is impacted by reduced sphingolipid content.

A number of hypothesised mechanisms for PM compartmentation have been investigated. Spira et al. (2012) found actin depolymerisation to have a minor impact on protein distribution, whereas enzymatic degradation of cell wall induced aggregation of membrane proteins into large patches. Reduced lipid complexity (following modification of either phospholipid, sphingolipid, or sterol content) was concluded to be strongly associated with reduced protein segregation. Membrane potential also appears to influence the lateral distribution of both sterols and proteins. Grossmann et al. (2007) applied a proton gradient uncoupler to yeast strains and found depolarisation of PM to result in dispersion of ergosterol and proteins from MCC patches. Notably, when cells were first treated with the sterol dye filipin and then with the proton gradient uncoupler, ergosterol did not disperse, and Can1 was no longer released from MCCs, suggesting that ergosterol has a structural role in maintaining MCC domains.

Overall, the work of Spira et al. (2012) and others indicate that ergosterol has a role in maintaining lateral compartmentation of the PM. Heterologous PM proteins produced in yeast may not localise to PM microdomains as anticipated in cells with altered sterol content, without further engineering. For example, Grossmann et al. (2008) report reduced localization of Hup1 (a hexose/H^+ symporter from *Chlorella kessleri*) at MCC domains of *S. cerevisiae erg2Δ*, *erg24Δ*, and *erg6Δ* strains. Further phenotypes of ergosterol biosynthesis mutants are likely to be explained in part by the altered activities of PM processes, which also have implications for yeast as microbial cell factories as discussed below.

4.2 | Vesicular trafficking

There are multiple vesicular trafficking pathways in yeast, which deliver proteins and other compounds between cellular compartments (reviewed in Feyder, De Craene, Bär, Bertazzi, & Friant, 2015). Proteins synthesized at the ER are trafficked via membrane-bound vesicles to the Golgi apparatus. At the Golgi, proteins are sorted for transport to either the PM via the secretory pathway or the vacuole via the vacuolar protein sorting pathway or alkaline phosphatase pathway. Meanwhile, PM proteins (and external compounds) are continuously internalised by endocytosis (reviewed in Goode, Eskin, & Wendland, 2015; Lu, Drubin, & Sun, 2016) and transported to endosomes, where they are sorted for transport to either the vacuole for degradation or the Golgi for recycling.

The efficacy of various vesicular transport pathways in ergosterol biosynthesis mutants has been summarised in Table 1. In general, the trafficking and maturation of most proteins does not appear to be strongly affected in viable *ergΔ* strains. For example, correct maturation of carboxypeptidase Y (indicating delivery from ER to Golgi and Golgi to vacuole) and Gas1 (indicating delivery from ER to Golgi), as well as secretion of invertase to periplasm has been reported for a number of *ergΔ* strains (Heese-Peck et al., 2002; Munn et al., 1999). However in *erg2Δ*, *erg3Δ*, and *erg6Δ* strains, the tryptophan permease Tat2 has been found to accumulate in vacuolar compartments, rather than MCC domains of the PM (Daicho et al., 2009; Estrada et al., 2015; Guan et al., 2009; Umebayashi & Nakano, 2003). Notably in wild type yeast cells, vacuolar accumulation of Tat2 occurs under conditions of reduced nutrient availability via the inactivation of Target Of Rapamycin Complex (TORC)1 kinase activity (Beck, Schmidt, & Hall, 1999; Schmidt, Beck, Koller, Kunz, & Hall, 1998).

The TORC1 complex functions as a master regulator of cell growth. Under conditions favourable for growth, TORC1 has kinase activity and promotes processes required for cell growth and division, such as ribosome biogenesis and translation initiation. Activation of TORC1 has been shown to promote the endocytosis of some transporters, such as Can1 (MacGurn, Hsu, Smolka, & Emr, 2011), and inhibit the endocytosis of other transporters, including Tat2 (De Craene, Soetens, & Andre, 2001; Schmidt et al., 1998). Vacuolar accumulation of Tat2 under normal growth conditions in certain *ergΔ* strains, is therefore suggestive of reduced TORC1 activity. In support of this, Estrada et al. (2015) found an *erg3Δ* strain to be more sensitive to treatment with the TORC1 inhibitor rapamycin, as determined by reduced growth in the presence of rapamycin and rapid dephosphorylation of the TORC1 target Sch9 when treated with rapamycin. These results suggest reduced TORC1 activity in *erg3Δ* under normal growth conditions; however, the results could potentially also be due to increased intracellular accumulation of rapamycin, if efflux of rapamycin is reduced in *erg3Δ* (see Section 4.4). The TORC1 complex is sited at vacuolar membranes, and vacuoles are highly fragmented in many *ergΔ* strains (Heese-Peck et al., 2002; Munn et al., 1999). It is therefore plausible that the regulation and activity of TORC1 could be perturbed in ergΔ strains. It is therefore plausible that the regulation and activity of TORC1 could be perturbed in *ergΔ* strains. Alternatively, TORC1 activity could be reduced in *ergΔ* strains in response to other physiological conditions that are unsupportive for growth; multiple signals including nutrient availability, salt stress, redox stress, and temperature stress regulate TORC1 activity; however, the mechanisms by which inputs are sensed and integrated remain to be elucidated (Loewith & Hall, 2011).

Yeast endocytosis is commonly investigated by studying uptake of the lipophilic dye FM4–64, the water-soluble dye Lucifer yellow (LY), and/or radio-labelled α-factor. Internalisation of FM4–64 has been observed for all *ergΔ* strains tested (Heese-Peck et al., 2002), indicating functional endocytosis of PM. However, defects have been observed in the internalisation of the mating pheromone α-factor and of the LY dye (Heese-Peck et al., 2002; Munn et al., 1999).

In wild type cells, the transmembrane receptor of α-factor, Ste2, undergoes a basal level of endocytosis in the absence of mating

TABLE 1 Membrane trafficking phenotypes of ergosterol pathway mutants

Similar to wild type; ✓. Slightly reduced; ↓. Reduced; ↓↓. Strongly reduced; ↓↓↓. Empty; not tested.

	erg6Δ	*erg2Δ*	*erg3Δ*	*erg5Δ*	*erg4Δ*	*erg2Δ erg6Δ*	*erg3Δ erg6Δ*	*erg2Δ erg3Δ*	*erg4Δ erg5Δ*	References
Protein maturation and plasma membrane delivery pathways										
Carboxypeptidase Y maturation (ER to Golgi, Golgi to vacuole)	✓	✓				✓				Munn et al. (1999)
Gas1 maturation (ER to Golgi)		✓	✓				✓			Heese-Peck et al. (2002)
Invertase secretion to periplasm	✓	✓				✓				Munn et al. (1999)
Localisation of Ste2 at plasma membrane						✓	✓			Heese-Peck et al. (2002)
Fus1-Mid1-GFP localised at plasma membrane	↓↓				↓↓					Proszynski et al. (2005)
Tat2 localised at plasma membrane	↓↓		↓↓							Estrada et al. (2015), Umebayashi and Nakano (2003)
Internalisation and post-internalisation pathways										
Internalisation of lipophilic dye FM4-64	✓	✓	✓			✓	✓	✓	✓	Heese-Peck et al. (2002)
Hyperphosphorylation and ubiquitination of Ste2 upon α-factor treatment			✓			↓↓↓	↓↓↓		✓	Heese-Peck et al. (2002)
Uptake of radio-labelled α-factor (24 °C)	↓	↓↓				↓↓↓				Munn et al. (1999), Heese-Peck et al. (2002)
Uptake of radio-labelled α-factor (37 °C)	↓↓	↓↓	✓			↓↓↓	↓↓↓	↓↓	✓	Munn et al. (1999), Heese-Peck et al. (2002)
Trafficking of water-soluble Lucifer Yellow dye to vacuole	✓	↓↓↓	↓↓↓			↓↓↓	↓↓↓	↓↓	↓	Munn et al. (1999), Heese-Peck et al. (2002)
Vacuole morphology	✓	↓↓↓	↓↓			↓↓	↓↓	↓↓↓	↓↓	Munn et al. (1999), Heese-Peck et al. (2002)
Vacuole morphology	↓↓↓		↓↓↓	↓↓↓	↓↓					Kato and Wickner (2001)
α-Factor degradation following trafficking to vacuole (37 °C)	✓	✓								Munn et al. (1999)

pheromone. Upon binding α-factor however, Ste2 is hyperphosphorylated, ubiquitinated, and the receptor-ligand complex is rapidly internalised (Toshima, Nakanishi, Mizuno, Toshima, & Drubin, 2009). Internalisation of α-factor has been found to be similar to wild type in *erg3Δ* and *erg4Δerg5Δ* strains, delayed in an *erg6Δ* strain, significantly reduced in *erg2Δ* and *erg2Δerg3Δ* strains, and strongly reduced in an *erg3Δerg6Δ* strain (Heese-Peck et al., 2002; Munn et al., 1999). In *erg2Δerg6Δ* and *erg3Δerg6Δ* strains with the most severe response, the Ste2 receptor was localised at the PM but not phosphorylated or ubiquitinated in response to α-factor (Heese-Peck et al., 2002), suggesting that the reduced internalisation observed in *ergΔ* strains could be a result of inefficient modification of Ste2 upon ligand binding. Notably, ergosterol has been found to interact with the kinase Yck2 (Li, Gianoulis, Yip, Gerstein, & Snyder, 2010), which has a role in Ste2 phosphorylation (Feng & Davis, 2000). It is possible that this kinase does not function efficiently in strains with modified sterol composition.

In strains where sufficient α-factor is internalised, degradation of the pheromone has been detected, indicating successful delivery to the vacuole (Munn et al., 1999). However, vacuolar accumulation of the water-soluble dye LY is significantly reduced in most *ergΔ* strains (Heese-Peck et al., 2002; Munn et al., 1999). Requirements for the internalisation of LY in fluid phase and the lipophilic dye FM4–64 (chemical structures shown in Figure 4), which binds membranes, may be different, or basal endocytosis levels may be reduced in *ergΔ* strains owing to altered Ypk1 activity. Ergosterol not only copurifies with the Ypk1 kinase from wild type *S. cerevisiae* cells but also augments Ypk1 kinase activity *in vitro* (Li et al., 2010). Additionally, Ypk1 isolated from *erg4Δ* cells exhibits five-fold less kinase activity than Ypk1 isolated from wild type cells. The relationship between Ypk1 and endocytosis is complex. Stimuli that increase PM tension (including hypotonic shock, mechanical stress, and inhibition of sphingolipid biosynthesis) induce TORC2-mediated activation of Ypk1 and a subsequent reduction in endocytosis rates, among other outputs (Berchtold et al., 2012; Gaubitz, Prouteau, Kusmider, & Loewith, 2016; Leskoske, Roelants, Marshall, Hill, & Thorner, 2017; Niles, Mogri, Hill, Vlahakis, & Powers, 2012; Roelants et al., 2017). However,

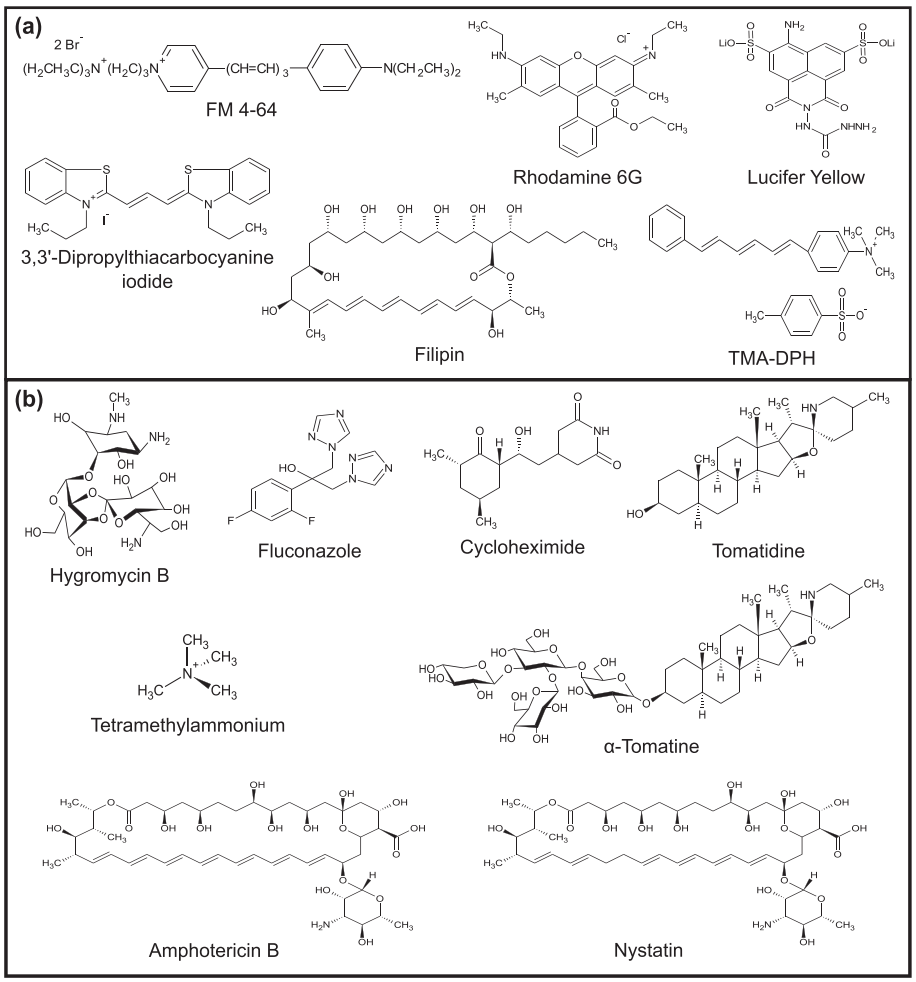

FIGURE 4 Structures of compounds applied to *Saccharomyces cerevisiae ergΔ* strains (a) Fluorescent dyes and markers referenced in this text. FM4–64; *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide. TMA-DPH; 1-[4-(trimethylamino)pheny]-6-phenyl-1,3,5-hexatriene. (b) Selected mycotoxic compounds to which *ergΔ* strains show altered tolerance

basal Ypk1 activity is also required for endocytosis. Yeast strains lacking Ypk1 fail to accumulate LY dye and exhibit reduced internalisation of α-factor (deHart, Schnell, Allen, & Hicke, 2002). Phosphorylation of Ypk1 by Pkh1 or its paralog Pkh2 (at a site distinct from those phosphorylated by TORC2) is required for both vacuolar accumulation of LY dye, and Ste2-mediated internalisation of α-factor (deHart et al., 2002). The reduced uptake of LY dye in *ergΔ* strains could therefore result from altered Ypk1 activity, although to the best of our knowledge, the activation state of Ypk1 in strains other than *erg4Δ* has not been assessed.

The reduced vacuolar fluorescence of LY dye observed in many *ergΔ* strains could potentially also be a result of reduced fusion between vacuolar compartments in the *ergΔ* strains. Although wild type yeast cells typically have one to three vacuolar compartments, many *ergΔ* strains contain highly fragmented vacuoles (Heese-Peck et al., 2002; Munn et al., 1999), with reduced fusion between vacuolar compartments detected *in vitro* and *in vivo* (Kato & Wickner, 2001). Experiments with isolated vacuoles have indicated that sterol deficiency disrupts the priming step of vacuole fusion, wherein SNARE (Soluble NSF Attachment Protein Receptor) and HOPS (Homotypic fusion and vacuole Protein Sorting) complexes are altered (Kato & Wickner, 2001). It is notable that in the studies by Munn et al. (1999) and Heese-Peck et al. (2002), *erg6Δ* was the only *ergΔ* strain that exhibited normal vacuole morphology and was also the only strain to accumulate LY dye similarly to wild type.

With regards to microbial cell factories, these studies indicate that in strains with altered sterol content, compounds are internalised by endocytosis at different rates. This is of relevance to microbial cell factory cultures in which chemicals are added to induce or suppress specific cellular processes (discussed further in Section 4.4). Meanwhile, the reduced accumulation of Tat2 (a high affinity tryptophan and tyrosine permease) at PM means that *ergΔ* strains may have higher requirements for tryptophan and tyrosine in growth media (Umebayashi & Nakano, 2003), and indicates that when strains are engineered to produce heterologous proteins, they may not be trafficked to the anticipated location. For example, Proszynski et al. (2005) report that in *erg6Δ* and *erg4Δ* strains, a GFP-tagged chimera of Fus2 and Mid2 protein domains accumulated in the Golgi network rather than at the PM. There are many instances where it may be desirable to target heterologous proteins to the PM of microbial cell factories. For example, yeast biosensors in which heterologous PM-localised receptors interface with native signalling pathways have been reported (reviewed in Adeniran, Sherer, & Tyo, 2015), and recently used to develop a bioassay for melatonin production (Shaw et al., 2019), as well as bioassays for human pathogens (Ostrov et al., 2017; Shaw et al., 2019). Additionally, heterologous transporters have

been expressed in yeast strains to improve salt tolerance and fermentation performance (Dibalova-Culakova, Alonso-Del-Real, Querol, & Sychrova, 2018). The subcellular location of heterologous transporters and other PM proteins should be verified in microbial cell factories with altered sterol content.

4.3 | Mating

The yeast mating response (reviewed in Bardwell, 2005) is initiated following detection of mating pheromone at the PM. In *S. cerevisiae*, MATa cells produce a-factor and detect α-factor at Ste2 receptors, whereas MATα cells produce α-factor and detect a-factor at Ste3 receptors. This double receptor system prevents cells from initiating mating in response to self. The transmembrane Ste2 and Ste3 proteins are G-protein coupled receptors. Ligand binding to pheromone receptor stimulates the Gα subunit of the associated G-protein to release Gβ and Gγ subunits, which recruit scaffold protein Ste5 and kinases Ste20, Ste11, Ste7, and Fus3 to the PM. A kinase cascade then results in activation of Far1 (which mediates cell cycle arrest) and Ste12 (which mediates activation of mating associated genes, including *FUS1*). Mating cells arrest their cell cycle and develop a schmoo morphology, as they project towards the detected mating partner. Upon contact, the cell walls of the mating pair fuse and are degraded, enabling the PMs to come into contact and fuse in a mechanism involving Fus1. Filipin staining has indicated that sterols are concentrated at the tip of schmoo mating projections and at sites of contact between mating pairs (Bagnat & Simons, 2002).

As discussed in Section 4.2, many *ergΔ* strains display reduced internalisation of α-factor. The development of schmoo morphology upon pheromone treatment indicates that the mating response is initiated in these strains (Heese-Peck et al., 2002), however downstream mating defects have been reported. For example, Jin et al. (2008) studied mating pairs of deletion mutants expressing GFP in MATa cells and red fluorescent protein in MATα cells. Mixing of fluorescent signals was used to indicate successful PM fusion between mating pairs. Strains lacking Erg2, Erg3, or Erg6 exhibited reduced occurrence of sterol accumulation at the mating projection tip, reduced expression from the *FUS1* gene promoter, and reduced occurrence of fusion. The *erg3Δ* mating pairs showed the strongest defect in membrane fusion and lowest level of *FUS1* expression, although this mutant was found to internalise α-factor similarly to wild type in the study by Heese-Peck et al. (2002). Localisation of GFP-Ste5 at the mating projection tip was also found to be reduced in the *erg3Δ* strain. The reduced accumulation of sterols and proteins involved in mating at the pheromone detection site, and reduced induction of *FUS1*, indicates defective pheromone response signalling in *erg2Δ*, *erg3Δ*, and *erg6Δ* strains.

In a similar experimental setup using mating pairs expressing GFP and mCherry, Aguilar et al. (2010) found pairs of *erg4Δ* mutants to be severely defective in mating, with reduced development of schmoo morphology and reduced occurrence of membrane fusion. In contrast to the phenotypes reported for *erg2Δ*, *erg3Δ*, and *erg6Δ* strains by Jin et al. (2008), the *erg4Δ* MATa cells underwent cell cycle arrest, induced *FUS1* expression and exhibited polarised distribution of sterols and proteins involved in mating upon α-factor treatment, although a previous study had reported lack of polarised distribution for Ste20 (Tiedje, Holland, Just, & Höfken, 2007). In the study by Aguilar et al. (2010), schmoo formation but not cell fusion was rescued by exogenously supplying ergosterol. Intriguingly, deletion of *ERG5* in *erg4Δ* cells restored both schmoo formation and membrane fusion between mating pairs. These results suggest that accumulation of the highly unsaturated ergosta-5,7,22,24(28)-tetraenol, which accumulates in the *erg4Δ* strain, restricts the ability of mating pairs to fuse.

With regards to the development of yeast microbial cell factories, these studies highlight that mating occurs with reduced efficiency in strains accumulating altered sterols. Therefore, mating as an engineering strategy should generally be avoided if sterol content has been altered. Additonally, biosensors that utilise pheromone response pathway components (Ostrov et al., 2017; Shaw et al., 2019) may not function as well in strains with altered sterol content.

Importantly, recent studies have utilised the yeast pheromone response to separate yeast growth from product generation. The delay of production until cells have grown to a particular cell density (reviewed in Venayak, Anesiadis, Cluett, & Mahadevan, 2015) is advantageous if transgene expression and/or the heterologous product is toxic or if production severely starves the cells of metabolites required for growth. Yu et al. (2017) for example, found separating *S. cerevisiae* growth from docosanol production to increase docosanol yield by four-fold. The engineering of yeast strains to produce mating pheromone of the opposite mating type, and activate pheromone response pathways at an appropriate cell density, has been proposed as a cost-effective means to separate growth from production (Williams, Nielsen, & Vickers, 2013), with the additional benefit that pheromone-mediated cell cycle arrest could result in increased flux to product rather than cell proliferation. Native and engineered pheromone-responsive promoters have been used to express genes of interest when sufficient pheromone accumulates in growth media (Williams et al., 2013), and repression of genes in response to pheromone accumulation has also been demonstrated, by introducing pheromone-responsive RNA interference (Williams et al., 2015). Williams, Peng, Vickers, and Nielsen (2016) have also explored the productivity of cells under pheromone-mediated cell cycle arrest, and suggest that yeast cells undergoing the mating response are in a metabolically active stationary phase, suitable for the production of a range of fine chemicals. However, our review of *ergΔ* strain phenotypes indicates that in some cases, use of the mating response may not be compatible with repression of ergosterol biosynthesis. This should be carefully considered prior to strain development.

4.4 | Tolerance to mycotoxic compounds

Examples of compounds that have been applied to *S. cerevisiae ergΔ* mutants are included in Figure 4, and the tolerances of *ergΔ* strains to various exogenous compounds are reported in Table 2.

TABLE 2 Tolerances of *ergΔ* strains relative to wild type

Arrows indicate increase (↑) or decrease (↓) in tolerance relative to wild type (WT). Double arrow indicates phenotype stronger than other *ergΔ* strains. As WT; tolerance similar to wild type.

	erg6Δ	*erg2Δ*	*erg3Δ*	*erg5Δ*	*erg4Δ*	*erg3Δ erg5Δ*	*erg3Δ erg4Δ*	*erg4Δ erg5Δ*	Reference
Chemical treatments									
Hygromycin B	↓	↓	slight ↓	as WT	↓				Kodedová and Sychrová (2015)
Hygromycin B	↓↓	↓↓	↓	↓	↓↓				Barreto et al. (2011)
Tetramethylammonium	↓↓	↓↓	↓	as WT	↓↓				Kodedová and Sychrová (2015)
Tetramethylammonium	↓↓	↓↓	↓	↓	↓↓				Barreto et al. (2011)
Spermine	↓↓	↓↓	↓	↓	slight↑				Barreto et al. (2011)
Cycloheximide	↓↓	↓↓	↓	↑	↓				Abe and Hiraki (2009)
Cycloheximide	↓	↓↓	↓		↓				Mukhopadhyay et al. (2004)
Clotrimazole	as WT	as WT	as WT	↓	as WT				Kodedová and Sychrová (2015)
Ketoconazole	↓	↓	as WT	as WT	↓				Kodedová and Sychrová (2015)
Fluconazole	as WT	↓↓	↓	↓↓	↓↓				Kodedová and Sychrová (2015)
Fluconazole	↑	slight↑	↑		slight↑				Mukhopadhyay et al. (2004)
Fluconazole			↑						Kontoyiannis (2000)
Fluconazole	↑	↓	↑						Bhattacharya et al. (2018)
Itraconazole	↓	↓↓	as WT	↓	as WT				Kodedová and Sychrová (2015)
Lovastatin	↓								Bhattacharya et al. (2018)
Fenpropimorph	↑	↓	↑						Bhattacharya et al. (2018)
o-Phenanthroline	↓	↓	↓		↓				Mukhopadhyay et al. (2004)
Sulfometuron methyl	↓	↓	↓		↓				Mukhopadhyay et al. (2004)
4-Nitroquinoline	↓	↓	↓		↓				Mukhopadhyay et al. (2004)
Methotrexate	↓	↓↓	↓↓		↓↓				Mukhopadhyay et al. (2004)
Amphotericin B	slight↑	slight↑		↑↑	slight↑				Gazdag et al. (2014)
Nystatin	↑	↑	as WT	as WT	↓				Kodedová and Sychrová (2015)
Nystatin	↑↑	↑↑	↑						Simons et al. (2006)
Nystatin	↑		↑	↑	↑				Bhattacharya et al. (2018)
α-Tomatine	↑	↓	↓						Simons et al. (2006)
Tomatidine	↑	as WT	↑↑						Simons et al. (2006)
Other conditions									
Heat (39.5 °C)		↑	↑↑	↑↑	↑	↑↑	↑	↑↑	Liu et al. (2017)
Low pH (2.75)		↓	↑	↑	↓	↓↓	as WT	as WT	Liu et al. (2017)
Ethanol (5% v/v)		as WT	slight↓	as WT	as WT	↓↓	slight↓	↑	Liu et al. (2017)
Ethanol (7% v/v)	↓								Inoue et al. (2000)
Lithium cations	↓	↓	↓	slight↓	↓				Kodedová and Sychrová (2015)
Lithium cations	↓								Welihinda et al. (1994)
High NaCl (1.2 M)	slight ↓	slight↓	slight↓	as WT	slight↓				Kodedová and Sychrová (2015)
High KCl (1 M)		as WT	slight↓	as WT	as WT	↓	as WT	as WT	Liu et al. (2017)
Low KCl (1 mM)	↓↓	↓↓	↓	↓	↓				Barreto et al (2011)
H_2O_2 (3 mM)		↑	↓	as WT	↓	↓	slight↓	↓	Liu et al. (2017)
H_2O_2	↓		↓						Branco et al. (2004)
Acetate (60 mM)		↓	↓↓	↓	↓↓	↓↓	↓↓	↓	Liu et al. (2017)
High glucose (10% w/v)		↓	↑	↑	↑	↑	↑	↑↑	Liu et al. (2017)
High glucose (2.5 M)	↓	↓	↓	as WT	↓				Kodedová and Sychrová (2015)
High sorbitol (2.8 M)	↓	↓	↓	as WT	↓				Kodedová and Sychrová (2015)
Low tryptophan	↓								Umebayashi and Nakano (2003)

As discussed in Section 4.2, the reduced vacuolar accumulation of LY dye in many *ergΔ* strains could be due to reduced rates of fluid phase endocytosis; however, in the development of antifungal pharmaceuticals, blocking ergosterol biosynthesis has been found to decrease yeast tolerance to a wide range of drugs. Sykes (1979) report increased susceptibility of *Candida albicans* and *Trichomonas vaginalis* to anisomycin, ascomycin, azalomycin F, brefeldin A, and copiamycin, when applied synergistically with imidazoles that inhibit ergosterol biosynthesis. Jensen-Pergakes et al. (1998) report removal of the *ERG6* gene in *C. albicans* to increase strain susceptibility to terbinafine, tridemorph, fenpropiomorph, fluphenazine, cycloheximide, cerulenin, and brefeldin A. Likewise, *S. cerevisiae* strains are usually tolerant to

brefeldin A (which in mammalian cells blocks secretion of proteins from the ER to Golgi); however, brefeldin A inhibits ER to Golgi transport in *S. cerevisiae erg6Δ* strains, with mycotoxic effect (Graham, Scott, & Emr, 1993; Vogel, Lee, Kirsch, Rose, & Sztul, 1993).

It has been hypothesised that suppressing ergosterol biosynthesis results in increased intracellular accumulation of exogenously applied chemicals, which reduces the strain's tolerance to mycotoxic compounds. As discussed below, this has been attributed to hyperpolarisation of the PM, increased membrane permeability, and decreased activity of efflux pumps such as Pdr5 (an ATP-binding cassette transporter, overexpression of which confers resistance to a broad range of compounds; Kolaczkowski et al., 1996).

The increased susceptibility of *ergΔ* mutants to cationic compounds, such as hygromycin B and trimethylammonium, has been partially attributed to an increase in membrane hyperpolarisation, as indicated by uptake of potentiometric dyes (Barreto et al., 2011; Kodedová & Sychrová, 2015), although the efflux of such dyes could be reduced in *ergΔ* strains (Kodedová & Sychrová, 2015). Meanwhile, Abe and Hiraki (2009) highlight a correlation between increased cycloheximide susceptibility, and the reduced membrane rigidity of different *ergΔ* strains, as indicated by anisotropy of the fluorescent dye TMA-DPH. Kaur and Bachhawat (1999) also report increased cycloheximide susceptibility for *erg6Δ*, *erg2Δ*, *erg3Δ*, and *erg4Δ* strains and investigated the efflux activity of Pdr5, for which cycloheximide is a substrate. Kaur and Bachhawat (1999) found the energy-dependent efflux of the fluorescent dye Rhodamine 6G (another known substrate for Pdr5), to be much slower than wild type in *erg4Δ*, *erg2Δ*, and *erg6Δ* strains. Efflux in the *erg3Δ* strain was also delayed but to a lesser extent than the other strains. Overexpression of *PDR5* increased cycloheximide tolerance in *erg4Δ*, *erg3Δ*, and to a lesser extent in *erg2Δ*. Further evidence for sterol composition impacting the activity of efflux pumps came from a study by Kontoyiannis (2000), in which the fluconazole resistance imparted by an activated allele of transcription factor gene *PDR1* (termed *PDR1–100*), resulting in increased *PDR5* transcription, was found to be diminished in the absence of Erg3.

In contrast to the reduced tolerance of *ergΔ* strains to many antifungal compounds, the enhanced fluconazole resistance of many *C. albicans* isolates has been attributed to a lack of Erg3 activity (Kelly et al., 1997; Kelly, Lamb, Corran, Baldwin, & Kelly, 1995; Martel et al., 2010; Sanglard, Ischer, Parkinson, Falconer, & Bille, 2003; Vale-Silva et al., 2012). Azoles including fluconazole inhibit Erg11 activity, and sterol profiling following fluconazole treatment has found that 14α-methyl-fecosterol accumulates in *erg3Δ* strains, whereas 14α-methylergosta8–24(28)dienol accumulates in strains with functional Erg3. It has therefore been hypothesised that accumulation of 14α-methylergosta8–24(28)dienol is detrimental to *C. albicans*. The relative tolerances of *S. cerevisiae ergΔ* strains to azole compounds have differed between reports and are summarised in Table 2.

Certain *ergΔ* strains also exhibit increased tolerance to antifungals that interact directly with sterols. For example, many *S. cerevisiae ergΔ* strains have been reported to exhibit enhanced tolerance to nystatin (Bhattacharya, Esquivel, & White, 2018; Kodedová & Sychrová, 2015; Simons et al., 2006). In an adaptive evolution experiment by Fryberg, Oehlschlager, and Unrau (1974), nystatin-resistant *S. cerevisiae* strains were found to accumulate 5,6-dihydroergsterol rather than ergosterol. Nystatin is a polyene that has been shown to bind ergosterol in synthetic liposomes and form pores in membranes at high concentrations (Coutinho, Silva, Fedorov, & Prieto, 2004). Amphotericin B (AmB) is another polyene with mycotoxic effect. Recent studies utilizing chemical derivatives of AmB, membrane permeabilisation assays, ergosterol-binding assays, and nuclear magnetic resonance spectroscopy, have indicated that AmB mycotoxicity is relayed primarily through sequestration of ergosterol into cell surface aggregates (Anderson et al., 2014; Gray et al., 2012; Palacios, Dailey, Siebert, Wilcock, & Burke, 2011). Strains deficient in Erg5 (and to a much lesser extent Erg2, Erg4, or Erg6) have increased tolerance to AmB (Gazdag et al., 2014). It is plausible that in the strains with enhanced polyene tolerance, the drug has reduced affinity for the sterols present.

Steroidal glycoalkaloids have also been reported to permeabilise membranes via interaction with sterols (Keukens et al., 1992). In a study by Simons et al. (2006), the steroidal glycoalkaloid α-tomatine was found to induce electrolyte leakage when applied to *S. cerevisiae*, whereas the aglycone tomatidine did not, and both compounds displayed mycotoxic effect. When the tolerances of *erg2Δ*, *erg3Δ*, and *erg6Δ* strains were investigated, *erg6Δ* was more tolerant to α-tomatine, whereas *erg2Δ* and *erg3Δ* were less tolerant. Conversely, the *erg3Δ* strain had greatly enhanced tolerance to the aglycone tomatidine. Again, differing affinities of these compounds for different sterols would explain the phenotypes observed.

During the fermentation of yeast cell factories, chemicals of varying biochemical properties may be added to cultures to induce, suppress, or augment particular cellular processes. For example, the auxin-inducible degron system from plants has been used to degrade target proteins in yeast (Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009); tetracycline and related compounds have been used to repress or activate genes (Bellí, Garí, Piedrafita, Aldea, & Herrero, 1998; Garí, Piedrafita, Aldea, & Herrero, 1997); and the hormone β-estradiol has been used for precise control of gene expression (Louvion, Havaux-Copf, & Picard, 1993; Ottoz, Rudolf, & Stelling, 2014). Chemicals may also be added to inhibit specific metabolic pathways, to increase the intracellular concentration of substrates for fine chemical biosynthesis. For example, lovastatin inhibits HMG-CoA reductase activity (Gardner, Shan, Matsuda, & Hampton, 2001), and terbinafine inhibits squalene epoxidase activity (Ryder, 1992), both of which result in accumulation of substrates for the synthesis of high-value terpenes. Lower concentrations of these expensive chemicals may be required for yeast strains with altered sterol composition, which could drastically reduce production costs. For example, Cdr1 (the *C. albicans* homologue of Pdr5) has been found to export β-estradiol (Krishnamurthy, Gupta, Snehlata, & Prasad, 1998), therefore *ergΔ* strains with reduced Pdr5 activity may accumulate β-estradiol (which currently costs approximately £1,700 per 100 g) at a faster rate than wild type. On the other hand, some feedstocks contain inhibitors to yeast growth, such as furfural and coniferyl alcohol

(Deparis, Claes, Foulquié-Moreno, & Thevelein, 2017), which may have an increased impact on strains with altered sterol content.

4.5 | Ion homeostasis and osmoregulation

Potassium is necessary for multiple aspects of yeast growth and survival, while sodium accumulation has toxic effects, through potassium displacement and induction of hyperosmotic shock. Yeast therefore maintain relatively high intracellular concentrations of potassium and low concentrations of sodium (reviewed; Ariño, Ramos, & Sychrova, 2019). Potassium is imported through high affinity transporters such as Trk1, driven by the membrane potential generated by PM H^+-ATPases, including Pma1. Cytosolic pH is regulated by the activity of both PM ATPases, which export protons from the cell, and vacuolar ATPases (V-ATPases), which acidify the vacuole (Martínez-Muñoz & Kane, 2008).

Pyranine and pHlourin fluorescence measurements indicate that intracellular pH is lower in strains with reduced ergosterol levels (Calahorra, Lozano, Sánchez, & Peña, 2011; Kodedová & Sychrová, 2015), and *ergΔ* strains have a reduced ability to control intracellular pH upon treatment with sodium chloride (Kodedová & Sychrová, 2015). The pH of vacuolar compartments in *erg2Δ*, *erg3Δ*, *erg6Δ*, and *erg24Δ* strains was reported to be higher than wild type by Zhang et al. (2010). In this study, vacuolar fragments isolated from an *erg24Δ* strain exhibited reduced V-ATPase activity. Fragmented vacuoles and perturbed activities at vacuolar membrane have also been reported in many *ergΔ* strains (Heese-Peck et al., 2002; Kato & Wickner, 2001).

A number of studies using the fluorescent potentiometric dye 3,3′-dipropylthiacarbocyanine iodide have indicated that reduced ergosterol content results in hyperpolarisation of the PM (Calahorra et al., 2011; Kodedová & Sychrová, 2015), although the enhanced accumulation of this dye could also result from reduced rates of efflux through multidrug resistant pumps (Kodedová & Sychrová, 2015). The increased susceptibility of *ergΔ* mutants to cations has been highlighted as another indicator of membrane hyperpolarisation. Welihinda et al. (1994) report *S. cerevisiae erg6Δ* to be less tolerant to lithium and sodium cations, associated with three- to four-fold higher rates of influx, while efflux rates remained similar to wild type. Kodedová and Sychrová (2015) have since reported *erg2Δ*, *erg3Δ*, *erg4Δ*, and *erg6Δ* strains to have reduced tolerance to high levels of lithium ions and sodium chloride, although an *erg5Δ* strain had similar tolerances to wild type in this study.

Meanwhile, *erg2Δ* and *erg6Δ* strains have been found to exhibit reduced growth in low potassium conditions, with *erg3Δ*, *erg4Δ*, and *erg5Δ* strains impacted to a lesser extent (Barreto et al., 2011). Confocal microscopy of Trk1-GFP has indicated reduced PM localisation of this high affinity potassium transporter in *erg6Δ* strains (Barreto et al., 2011). Therefore, the reduced growth in low potassium reported for certain *ergΔ* mutants could be due to reduced presence of potassium transporters at the PM.

Some *ergΔ* strains have also been found to be less tolerant to treatment with high concentrations of glucose or sorbitol, which would induce osmotic pressure (Kodedová & Sychrová, 2015). In addition to potentially being more susceptible to osmotic stress, due to disrupted ion homeostasis, *ergΔ* strains may exhibit defects in their response to osmotic stress. The kinase Ssk22 is involved in relaying the osmotic stress response in yeast, as part of a MAP kinase cascade that activates Hog1 (Posas et al., 1996), and studies by Li et al. (2010) indicate a role for ergosterol in maintaining Ssk22 protein levels. Conversely, the hyperosmotic stress response in yeast involves rapid repression of *ERG2* and *ERG11* (mediated by the MAP kinase Hog1), and overexpression of the ergosterol pathway confers increased susceptibility to salt stress (Bhattacharya et al., 2018; Montañés, Pascual-Ahuir, & Proft, 2011). These cellular responses are coherent when considering the wider context of the hyperosmotic stress response, which involves cell cycle arrest (Clotet et al., 2006; Escoté, Zapater, Clotet, & Posas, 2004) and diversion of metabolic resources towards glycerol biosynthesis (reviewed in Hohmann, 2002).

These studies suggest that careful design of growth media is required in fermentations using strains with altered sterol content. For example, such strains may be less tolerant to high sugar and salt concentrations and may also require higher concentrations of potassium. Alternatively, strains may be further engineered to increase ion homeostasis (Deparis et al., 2017). The latter may be of preference; in first generation bioethanol production (utilising food crop biomass), streams of 35% sugar are used in order to attain ethanol titres of 16–18% (Deparis et al., 2017). In second generation bioethanol production (utilising nonfood crops as feedstock), fermentations are usually limited to 12% sugar; however, high concentrations of sodium salt may be present, due to feedstock pretreatment conditions and/or accumulation of sodium salt in pipelines and fermenters (Deparis et al., 2017). Certain feedstocks also contain metal cations, to which *ergΔ* strains may be more susceptible. For example, sugarcane bagasse usually contains Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} (Deparis et al., 2017).

4.6 | Ethanol tolerance

Many yeast including *S. cerevisiae* rapidly convert sugars to ethanol, as a means to outcompete competitors in sugar-rich environments (Dashko, Zhou, Compagno, & Piškur, 2014). Such yeasts are able to withstand ethanol concentrations that would be lethal to other microorganisms. However, higher ethanol concentrations are detrimental to yeast, and under certain circumstances fermentations can become "stuck" despite sugar still being available. Ethanol is considered to intercalate at the lipid-water interface of bilayers, increasing lipid head group spacing, and increasing the fluidity and ion permeability of membranes. At high ethanol concentrations, lipid bilayers become interdigitated, reducing membrane thickness by up to 30%, and disrupting PM processes including amino acid and glucose uptake (reviewed in Henderson & Block, 2014).

Experiments with model membranes have demonstrated that ergosterol is highly effective at reducing interdigitation of lipid bilayers in the presence of ethanol (Dickey, Yim, Yim, & Faller, 2009;

Tierney, Block, & Longo, 2005; Vanegas, Contreras, Faller, & Longo, 2012; Vanegas, Faller, & Longo, 2010). Elevated levels of unsaturated fatty acids and ergosterol have been associated with increased ethanol tolerance in a number of studies (reviewed in Henderson & Block, 2014). For example, Aguilera, Peinado, Millán, Ortega, and Mauricio (2006) compared the ethanol tolerance and lipid composition of five *S. cerevisiae* strains and found increased ergosterol, oleic acid, and palmitoleic acid content to be highly correlated with ethanol tolerance. The hypothesised role of ergosterol in ethanol tolerance is supported by the decreased ethanol tolerance of certain *erg*Δ strains. In a study by Liu et al. (2017), an *erg3*Δ*erg5*Δ strain was found to grow at 35% the rate of a wild type strain in the presence of 5% (v/v) ethanol, whereas the individual *erg3*Δ and *erg5*Δ strains grew similarly to wild type. Inoue et al. (2000) determined a strain unable to grow in 7% (v/v) ethanol to be deficient in Erg6. Compared with ergosterol, these *erg*Δ strains accumulate sterols with altered aliphatic tails; the *erg3*Δ*erg5*Δ strain accumulates sterols like ergosta-7-enol and episterol, with highly saturated aliphatic tails, whereas the *erg6*Δ strain lacks methyl substitution in its sterol aliphatic tails.

The increased susceptibility of certain *erg*Δ strains to ethanol should therefore be considered when engineering strains for bioethanol production, where high ethanol concentrations are required for efficient extraction (Abdel-Banat, Hoshida, Ano, Nonklang, & Akada, 2010). A minimum concentration of 4–5% (v/v) ethanol is usually required for ethanol distillation to be economically viable, and corn ethanol production often reaches final ethanol titres of 16–18% (Deparis et al., 2017). High ethanol concentrations may also be reached in the production of fine chemicals, when sugar-rich growth media is used. Many target genes for increasing ethanol tolerance have been identified over the years (reviewed in Deparis et al., 2017), and these findings could be utilised to increase the ethanol tolerance of microbial cell factories.

4.7 | High temperature

Although both high temperature and ethanol stress increase membrane fluidity and show an overlap in stress response pathways, the tolerance of *erg*Δ mutants to these stresses is very different. In an adaptive laboratory evolution experiment by Caspeta et al. (2014), nonsense mutations in *ERG3* were identified in all seven evolved strains with increased thermotolerance. To determine the impact of Erg3 deficiency, one of the identified mutations was reconstructed in the parental strain, and determined to confer 86% of the evolved strain's thermotolerant phenotype. The thermotolerant strains accumulated fecosterol under the culture conditions tested, with overall sterol content similar to the parental strain. The authors postulated that bended sterols are able to better maintain membrane dynamics at higher temperatures. Transcriptomics of the thermotolerant strains has also indicated, however, that these strains activate a heat stress response at lower temperatures and so are primed for growth at higher temperatures (Caspeta, Chen, & Nielsen, 2016). Liu et al. (2017) subsequently compared the thermotolerance of *erg2*Δ, *erg3*Δ, *erg4*Δ, *erg5*Δ, *erg3*Δ*erg4*Δ, *erg3*Δ*erg5*Δ, and *erg4*Δ*erg5*Δ strains and found them to exhibit a higher growth rate than wild type at 39.5°C. Of the individual mutants, *erg3*Δ and *erg5*Δ had the highest thermotolerance, and these mutations had an additive effect, with the *erg3*Δ*erg5*Δ strain showing 2.24-fold increase in growth rate relative to wild type at 39.5°C (Liu et al., 2017).

As explored by Abdel-Banat et al. (2010), there are many potential cost savings associated with increasing the thermotolerance of microbial cell factories. In the production of ethanol from starch for example, starch is liquefied at 90°C with a thermostable α-amylase, cooled for either separate or simultaneous saccharification and fermentation (32–35°C), and then heated again for ethanol extraction. During the fermentation phase, cooling is also required, as fermentations generate heat (Deparis et al., 2017). Abdel-Banat et al. (2010) estimate that for a 30,000-kL scale ethanol plant, a 5°C increase in fermentation temperature would save approximately US $30,000 per year in cooling costs, and up to US $250,000 per year in expenditure on hydrolase enzymes applied during fermentation, as most biomass-hydrolysing enzymes function optimally between 40°C and 50°C (Choudhary, Singh, & Nain, 2016). For example, the glucoamylase cocktail OPTIDEX L-300 (Genencor International, Inc.) has a two-fold higher activity at 40°C compared with 32°C (Abdel-Banat et al., 2010). Additionally, Abdel-Banat et al. (2010) demonstrate efficient vacuum extraction of ethanol at 40°C, but not at 35°C, suggesting that simultaneous fermentation and ethanol extraction by vacuum extraction may be feasible with higher temperature fermentations. In addition to reducing heating costs, simultaneous fermentation and ethanol extraction would be of benefit as ethanol has an inhibitory effect on yeast growth rate.

To test the translatability of their findings to other fungal species, Liu et al. (2017) removed the ortholog of *S. cerevisiae* Erg5 from the filamentous fungus *Penicillium oxalicum*, and found the mutant strain to exhibit significantly improved growth at 37°C relative to wild type. This is of industrial relevance as *P. oxalicum* grows optimally at 30°C but secretes cellulases that function optimally at 50°C. Hence, modification of sterol content could be a promising approach to develop a range of fungal strains for high-temperature fermentation.

5 | CONCLUSIONS

The viable mutants of the ergosterol biosynthesis pathway exhibit a range of phenotypes, including altered localisation of specific PM proteins, fragmentation of vacuoles, reduced completion of mating, altered susceptibility to chemical treatment, PM hyperpolarisation, increased tolerance to high temperatures, and reduced tolerance to other stresses such as high ethanol, salt and solute concentrations.

Notably there are differences in the severity of phenotype between different *erg*Δ mutants, reflecting the impact of different sterol compositions. The removal of methyl groups from the carbon 4 and carbon 14 of lanosterol would appear to be critical for sterol function, as strains deficient in Erg11, Erg25, Erg26, Erg27 or Erg28

are non-viable, although interestingly the *erg24Δ* strain can grow under specific conditions (Crowley et al., 1996).

Of the viable *ergΔ* mutants, strains lacking Erg2 often have the strongest phenotype. Time-resolved fluorescence anisotropy measurements indicate that there is greatly elevated freedom of rotational movement within the PM of this strain, and increased disorder (Abe & Hiraki, 2009). The *erg2Δ* strain accumulates sterols with unsaturation in carbon position 8(9), which differs considerably from the carbon 7 (8) unsaturation in ergosterol (Figure 2). These altered sterols could account for the striking differences in PM dynamics. Meanwhile, the lack of a carbon 24 methyl group in *erg6Δ* strains, is likely to alter interactions of the sterol side chain with fatty acid tails within the hydrophobic core of lipid bilayers.

In some instances, the accumulation of a specific sterol structure is hypothesised to impact a specific process. For example, the accumulation of ergosta-5,7,22,24(28)-tetraenol in *erg4Δ* strains is postulated to impede membrane fusion between mating cells (Aguilar et al., 2010). As discussed in section 4.3, the aliphatic tail of this sterol is particularly rigid due to the presence of two double-bonds.

Many of the phenotypes observed are due to altered processes at cell membranes. For example, reduced activity of efflux pumps and reduced V-ATPase activity. Multiple studies indicate that signalling processes are disrupted or respond differently in strains with altered sterol content. For example, the reduced phosphorylation of Ste2 in the presence of mating pheromone (Heese-Peck et al., 2002), and the trafficking of Tat2 to vacuoles as opposed to the PM (Estrada et al., 2015). Notably, in the analysis of metabolites that co-purify with kinases, ergosterol was found to co-purify with 15 different kinases (Li et al., 2010), including Ypk1 which regulates rates of endocytosis (deHart et al., 2002), Yck2 which is involved in Ste3 phosphorylation (Feng & Davis, 2000), and Ssk2 which is involved in responding to osmotic stress (Posas et al., 1996). Furthermore, ergosterol was found to augment Ypk1 kinase activity *in vitro* (Li et al., 2010), suggesting that yeast sterols could have a physiological role in facilitating the activity of some kinases.

The implications of *ergΔ* phenotypes in the development of yeast as microbial cell factories are many. In some cases, removing specific ergosterol biosynthesis genes may be beneficial. For example, when increased susceptibility to an expensive drug is desirable, or if it is optimal for fermentations to take place at higher temperatures. It may also be beneficial to suppress ergosterol biosynthesis in order to maximize flux towards an alternate pathway. Notably, suppression of specific enzymes in ergosterol biosynthesis has been found to increase expression of most genes in the ergosterol pathway (Caspeta et al., 2014), potentially increasing the availability of precursors of interest. However, there are important considerations to factor into the design of growth media when sterol profiles are altered; namely, the reduced tolerances of strains with altered sterol content to osmotic stress, high ethanol content, and low concentrations of specific amino acids and ions.

In conclusion, there are many potential benefits to modifying sterol composition in yeast cell factories, however the phenotypes of strains with altered sterol content are wide-ranging and should be considered when engineering strain and culture conditions in order to maximize productivity.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare. In this manuscript, we provide an unbiassed review of the literature relating to ergosterol biosynthesis and the phenotypes of yeast strains with altered sterol content.

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